Coral Reefs (2007) 26:189–197
DOI 10.1007/s00338-006-0183-9

REPORT

Reproductive periodicity and steroid hormone profiles in the sex-changing coral-reef fish, *Plectropomus leopardus*

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Received: 5 July 2006 / Accepted: 20 November 2006 / Published online: 6 February 2007

Abstract The reproductive biology of coral trout, *Plectropomus leopardus*, from the Great Barrier Reef (Australia) was investigated by correlating gonadal condition with plasma levels of gonadal steroids. Female fish were found to be regressed from mid-summer to early spring, after which rapid and cyclical increases in gonado-somatic index (I_G), maximum oocyte diameter (MOD) and plasma concentrations of estradiol-17β and testosterone were detected. Male fish, in contrast, commenced recrudescence slightly earlier in winter and responded with less dramatic increases in both I_G and plasma concentrations of testosterone and 11-ketotestosterone. The mode of oocyte development was multiple group-synchronous, and cyclical fluctuations in reproductive parameters (I_G, MOD and gonadal steroid concentrations) were synchronized with new-moon lunar phases. It is likely, therefore, that individual *P. leopardus* have the capacity to spawn on multiple occasions, with lunar periodicity. However, evidence suggests that early bouts of reproduction may be more important in terms of reproductive investment than subsequent bouts later in the same season. It is concluded that patterns of gametogenesis and steroidogenesis in *P. leopardus* are similar to the patterns displayed by other tropical groupers, suggesting that management regimes and propagation protocols developed for these fishes may also be appropriate for use with *P. leopardus*.

Keywords Serranidae · Coral trout · Protogynous hermaphrodite · Reproductive development · Sex-change · Lunar periodicity

Communicated by Biology Editor H.R. Lasker.

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Introduction

A large proportion of coral-reef fishes are sequentially hermaphroditic (i.e., sex-changing) (Reinboth 1988). Among these, some mature as female and later change sex to male (protogyny), while others mature as male and later change sex to female (protandry). Unlike the 'straightforward' sexual maturation of gonochoristic (fixed-sex) fishes, maturation in sequentially hermaphroditic fishes requires the (more or less) simultaneous quiescence and recrudescence of alternate (i.e., male and female) gonadal states (Frisch 2004). For this reason, reproductive development in sequentially hermaphroditic fishes is generally more complicated and less understood than it is in gonochoristic fishes.

To date, there have been comparatively few investigations of reproductive physiology in sequentially hermaphroditic fishes, especially those from tropical regions (Frisch 2004). However, many of these species support important reef fisheries, where the impact of fishing is typically directed toward larger size-classes

which, in the case of groupers (family Serranidae), can be comprised exclusively of male fish (Coleman et al. 1996; Adams et al. 2000). Understanding the effects of fishing-mediated alterations in the sex structure and demography of exploited fish populations requires a detailed understanding of the inter-relationships between fish size, sexual status, and the physiological regulation of sex-change and gametogenesis (Levin and Grimes 2002; Rowe and Hutchings 2003). This information can be used to predict (1) population responses to size-selective fishing, and (2) the resilience of size-truncated populations to further exploitation (Rowe and Hutchings 2003).

The coral trout (*Plectropomus leopardus*: Serranidae), otherwise known as leopard coral grouper, is a protogynous coral-reef fish. It is a major target of artisanal, recreational and commercial reef fisheries operating throughout the Indo-West Pacific region, including Australia's Great Barrier Reef (GBR) (Kailola et al. 1993; Sadovy et al. 2003). Much of the commercial catch is exported to Hong Kong, where the retail price of live specimens ranges from US$50 to $75 per kg (Sadovy et al. 2003). This high value has resulted in a strong demand for *P. leopardus* and an upward trajectory in total annual harvest (Williams 2002). Despite this trend, mounting evidence suggests that fishing has significantly decreased the abundance of *P. leopardus* in parts of Australia and South-East Asia (Sadovy et al. 2003; Williamson et al. 2004), thus emphasizing the need to develop sustainable management regimes and culture techniques for this species. Understandably, the reproductive biology of *P. leopardus* has become the subject of considerable attention in recent years. However, only descriptive aspects of gonad morphology have been published to date (Ferreira 1995; Samoilys and Roelofs 2000; Adams 2003), with all aspects of reproductive physiology and endocrinology yet to be addressed.

This study describes the temporal sequence of gametogenesis and steroidogenesis in male, female and transitional (i.e., sex-changing) *P. leopardus*. Plasma concentrations of the steroid hormones estradiol-17β (E_2), testosterone (T) and 11-ketotestosterone (11KT) were measured in view of their roles in the regulation of vitellogenesis (E_2), oogenesis (E_2 and T) and spermatogenesis (T and 11KT) in other teleosts (Pankhurst and Carragher 1991; Kime 1993; Pankhurst 2006). Sampling was aligned with the lunar cycle, based on the periodicity of spawning aggregations reported previously (Samoilys 1997). Furthermore, sampling was conducted over one calendar year to encompass an entire episode of gonadal recrudescence, serial spawning, and subsequent quiescence.

Materials and methods

Field sampling

One hundred and seventy-three coral trout were collected from coral reefs adjacent to Townsville, Australia (18°20′S, 146°45′E) during eleven sampling events spread across 1 year. Samples were collected every 3 months during periods of reproductive quiescence (autumn and winter), and monthly or bimonthly during periods of reproductive activity (spring and summer). Samples were collected as close as possible to the new-moon lunar phase, except on two occasions (October and November) when additional samples were collected between new-moon phases (Table 1). Surface seawater temperatures were determined at the time of sampling (±0.1°C) with a digital thermometer (Table 1).

Ten to 19 fish were captured during each sampling event (Table 1), with individual captures spread more or less evenly throughout the day (0800–1800 hour). Fish were captured by angling (i.e., a baited 8/0 hook attached to a 0.9 mm nylon fishing-line), which has a size selectivity of 50% at 32 cm Total Length (TL) and 100% over 39 cm (TL) (Fulton et al. 1999). Coral trout mature (100% of individuals) at ~36 cm (TL) (Adams et al. 2000) and grow to 70 cm (TL) (Heemstra and Randall 1993), thus indicating that the majority of mature fish were available to the sampling gear.

Upon capture, each fish was restrained in a foam cradle and a blood sample (2–4 ml) was collected from the caudal vasculature with a heparinized, hypodermic needle (22 gauge). Blood was transferred immediately to a plastic vial, centrifuged at 3,000×*g* for 5 min, and the plasma stored on ice (as per Frisch and Anderson 2005). TL was measured (±1 mm) with a standard rule, and total body weight estimated from published length–length and length–weight relationships (Heemstra and Randall 1993; Ferreira and Russ 1994). Gonads were removed by dissection and fixed in a solution of formaldehyde (9%), acetic acid (4%) and calcium chloride (1%) (as per Adams 2003). Upon return to the laboratory (*ca.* 12 h), plasma was centrifuged again (10,000×*g* for 5 min) and stored at −80°C until assay.

Gonadal development

Fixed gonads were blot-dried and weighed to the nearest 0.01 g. Gonado-somatic index (I_G) was calculated as gonad weight/total body weight × 100 (West 1990). A single transverse segment was cut from the medial region of one gonadal lobe for histological analysis

Table 1 The timing of sampling events (and the number of fish collected) in relation to new-moon lunar phases and surface seawater temperatures on the Great Barrier Reef (January 2004–January 2005)

Sampling date	New-moon date[a]	Temperature (°C)	Number of fish collected		
			Female	Transitional	Male
January 19	January 21	29.4	11	2	4
April 12	April 19	27.0	16	0	3
July 15	July 17	24.5	12	1	4
August 13	August 16	24.3	13	1	3
September 9	September 14	24.5	10	2	3
October 9	October 14	25.8	12	2	4
October 23	–	26.8	8	3	3
November 10	November 12	27.3	10	0	3
November 20	–	27.9	13	2	3
December 7	December 12	28.3	12	1	2
January 14	January 10	29.5	8	0	2

In Australia, spring and summer extend from September to November and December to February, respectively

[a] Data provided by Geosciences Australia

Table 2 Macroscopic and histological characteristics of female, transitional and male developmental stages of coral trout, *Plectropomus leopardus*

Developmental stage	Symbol	Defining characteristics
Immature female	F1	Ovary small with compact lamellae, each containing pre-vitellogenic oocytes (i.e., chromatin nucleolus and perinucleolus stages). Brown-bodies[a] absent and gonad wall is thin
Pre-vitellogenic female	F2	Lamellae less compact than F1, but contain oocytes of the same stages. Brown-bodies[a] present and gonad wall is thick
Vitellogenic female	F3	Overy large with vitellogenic oocytes (yolk vessicle, yolk globule, or migratory nucleus stages). Brown-bodies[a] sometimes present
Hydrated female	F4	Very large ovary containing hydrated oocytes. Post-ovulatory follicles sometimes present
Post-spawning female	F5	Lamellae disorganized and oocytes atretic. Brown-bodies[a] often present. Extensive vascularization
Early transitional	T1	Gonad dominated by ovarian lamellae (oocytes vitellogenic or pre-vitellogenic), but testicular tissue present in small crypts. Spermatocytes and spermatids present, but spermatozoa absent. Brown-bodies[a] often present
Late transitional	T2	Gonad contains ovarian lamellae (oocytes vitellogenic or pre-vitellogenic) and large crypts of testicular tissue. Spermatocytes and spermatids present, but spermatozoa absent. Brown-bodies[a] often present
Immature male	M1	Gonad small and dominated by testicular lobules (>50% of cross-sectional area), although some pre-vitellogenic oocytes may remain. Spermatozoa uncommon and sperm sinuses undeveloped
Mature male	M2	Gonad similar to M1, except crypts of spermatozoa are common, and sperm sinuses are well developed and filled with Spermatozoa

Adapted from Ferreira (1995) and Samoilys and Roelofs (2000)

[a] Atretic pre-ovulatory follicles

[previous studies found little difference between proximal, medial or distal regions of the gonad, and no difference between left or right gonadal lobes: Ferreira (1995); Samoilys and Roelofs (2000)]. Each segment was subsequently embedded in paraffin wax, sectioned at 5 μm, and mounted on a glass slide for staining with Mayer's haematoxylin and eosin (Adams 2003). Slides were examined in random order (to avoid possible biases in interpretation associated with prior knowledge of collection time and fish size) using a high-power microscope, and gonads were assigned to a developmental stage (Table 2) based on the most advanced male or female germ cells present (West 1990). Maximum oocyte diameter (MOD) was measured with an eyepiece micrometer (200× magnification) and averaged across ten of the largest oocytes present in each section (West 1990).

Steroid hormone analysis

Plasma concentrations of E_2, T and 11KT were measured by radioimmunoassay (RIA) following extraction from plasma with ethyl acetate using the reagents and protocols described by Pankhurst and Carragher (1992) (for E_2 and T) or Pankhurst and Kime (1991) (for 11KT). Extraction efficiency (mean recovery of

[^{3}H]-labeled steroid from triplicates of a plasma pool) was 94% for E_2, 91% for T and 61% for 11KT, and assay values for each steroid were adjusted accordingly. Assay specificity was verified by confirming parallelism in the binding curves of serially diluted plasma extracts and steroid standards. Inter-assay variability (%CV) measured from aliquots of a pooled standard was as follows: E_2 10.6% ($n = 3$); T 12.9% ($n = 3$); 11KT 11.1% ($n = 4$). The minimum detectable plasma concentration for each assay was 0.075 ng ml^{-1}.

Statistical analysis

Homoscedastic data were analyzed by one-way analysis of variance (ANOVA) followed by post hoc comparisons of group means using Tukey's HSD test (Zar 1999). Heteroscedastic data were transformed ($\log_{10}[x + 1]$) and then analyzed as above. In cases where transformation failed to correct heteroscedasticity, a Kruskal–Wallis test was used (Zar 1999). All analyses were performed using SPSS software (Chicago, IL, USA) and a significant difference was considered to exist if $p < 0.05$. All data given in the text and figures are the arithmetic mean ± standard error (SE) of untransformed data.

Results

The sex-ratio of captured fish was 3.7 females to each male (125/34), excluding transitional fish (14), which accounted for ~8% of the total catch. Female and male fish dominated the smaller and larger size classes (respectively), while transitional fish were distributed across a range of size classes (Fig. 1). Although sample sizes were small, there was no apparent relationship between the abundance of transitional fish and time of year (Table 1).

All female fish captured between January and July were found to be immature (F1) or pre-vitellogenic (F2). Female fish possessing vitellogenic oocytes (F3) were not captured until August, although the proportion of fish at this developmental stage increased dramatically thereafter and remained elevated until December (Fig. 2). Female fish with hydrated oocytes (F4) were captured during, but not between, the new-moon periods of October, November and December. Post-spawning female fish (F5) were captured both during and between the new-moon periods of October and November (Fig. 2).

Maximum oocyte diameter increased dramatically in early spring (September and October) and remained elevated until early summer (December) ($p < 0.001$),

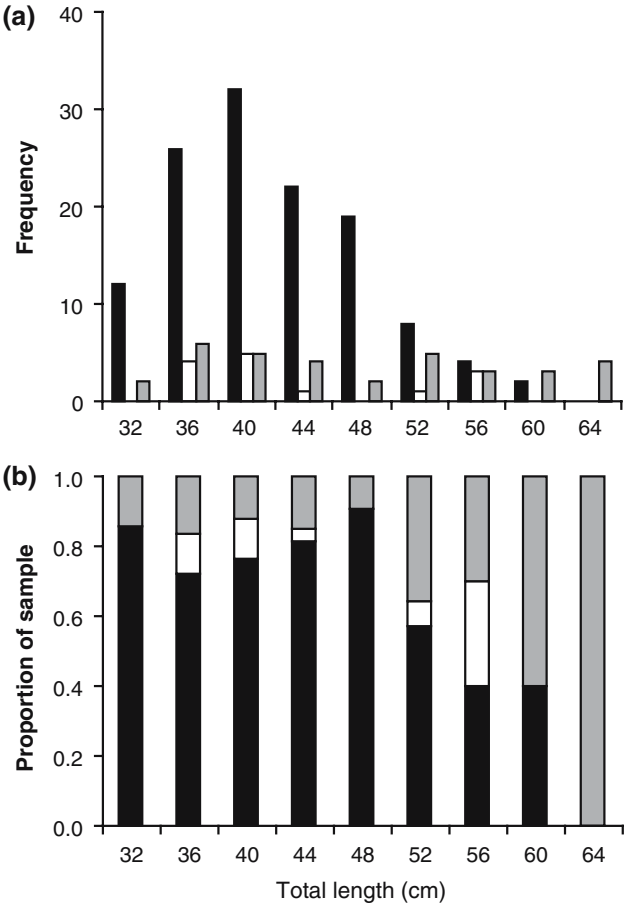

Fig. 1 The frequency (**a**) and proportion (**b**) of female (*black bars*), transitional (*white bars*) and male (*gray bars*) *Plectropomus leopardus* that were captured by angling on the Great Barrier Reef

although a reduction in MOD was observed in-between the new-moon periods (Fig. 3a). The same pattern of change was observed in I_G of female fish ($p < 0.001$) (Fig. 3b). By comparison, I_G in male fish was more stable (Fig. 3c), although increases in I_G during spring and summer (September–December) were also statistically significant ($p = 0.013$). Increases in I_G among female fish were approximately an order of magnitude greater than increases in I_G among male fish (cf. Fig. 3b, c).

Steroid hormone concentrations in the plasma of female fish were low throughout autumn and winter (March–August), although statistically significant increases in E_2 ($p < 0.001$) and T ($p < 0.001$) were observed after this time (Fig. 4a). Furthermore, E_2 and T followed the same cyclical pattern as MOD and I_G during spring and early summer (cf. Figs. 3a, b, 4a). In contrast, plasma concentrations of 11KT in female fish were consistently low (but detectable) throughout the year.

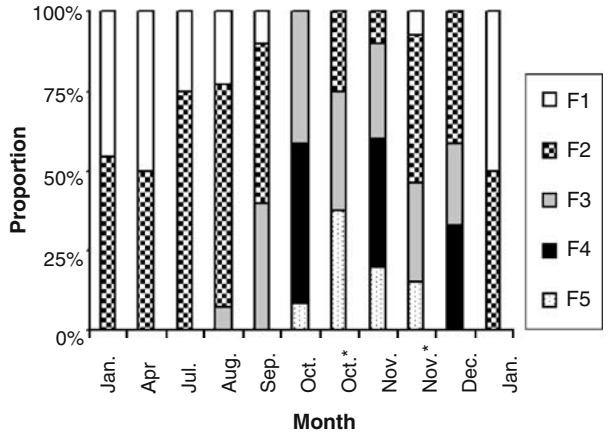

Fig. 2 Distribution of developmental stages among female *Plectropomus leopardus* that were captured over 1 year (2004–2005). Most samples were collected as close as possible to the new-moon lunar phase. Samples marked with an *asterisk* were collected between new-moon lunar phases. See Table 1 for sample sizes and Table 2 for definitions of developmental stages

There were no significant changes in plasma concentrations of T or 11KT in male fish during the year (Fig. 4b). However, plasma concentrations of both steroids were generally higher during spring and early summer (October–December). Estradiol-17β appeared to be absent from male fish, with plasma concentrations at or below the minimum detectable level.

When the data for successive new-moon phases in October, November, and December were compared in isolation (to permit parametric analyses), female fish were observed to experience a significant decline in I_G ($p = 0.046$), T concentration ($p < 0.002$) and E_2 concentration ($p < 0.001$), but not in MOD. The decline in I_G was probably associated with the appearance of previtellogenic female fish (F2) in the samples, because mean I_G of vitellogenic (F3) female fish was not significantly different during successive new-moon phases. The same was true for hydrated female fish (F4).

Immature (F1) and pre-vitellogenic (F2) female fish were characterized by low concentrations of all steroids in the plasma (Fig. 5a). In contrast, plasma concentrations of both E_2 and T were elevated in vitellogenic (F3) and hydrated (F4) fish ($p < 0.01$ and $p < 0.001$, respectively). Post-spawning fish (F5) had intermediate concentrations of T (i.e., not statistically different from T concentrations in other female fish) and low concentrations of E_2.

Transitional fish (particularly T1) were characterized by low (but detectable) concentrations of E_2, T and 11KT in the plasma (Fig. 5b). In contrast, male fish (M1 and M2) generally had higher concentrations of plasma T and 11KT, although only the latter was statistically different from transitional fish ($p < 0.02$). In general terms, levels of plasma T and 11KT increased concomitantly with the proportion of testicular tissue

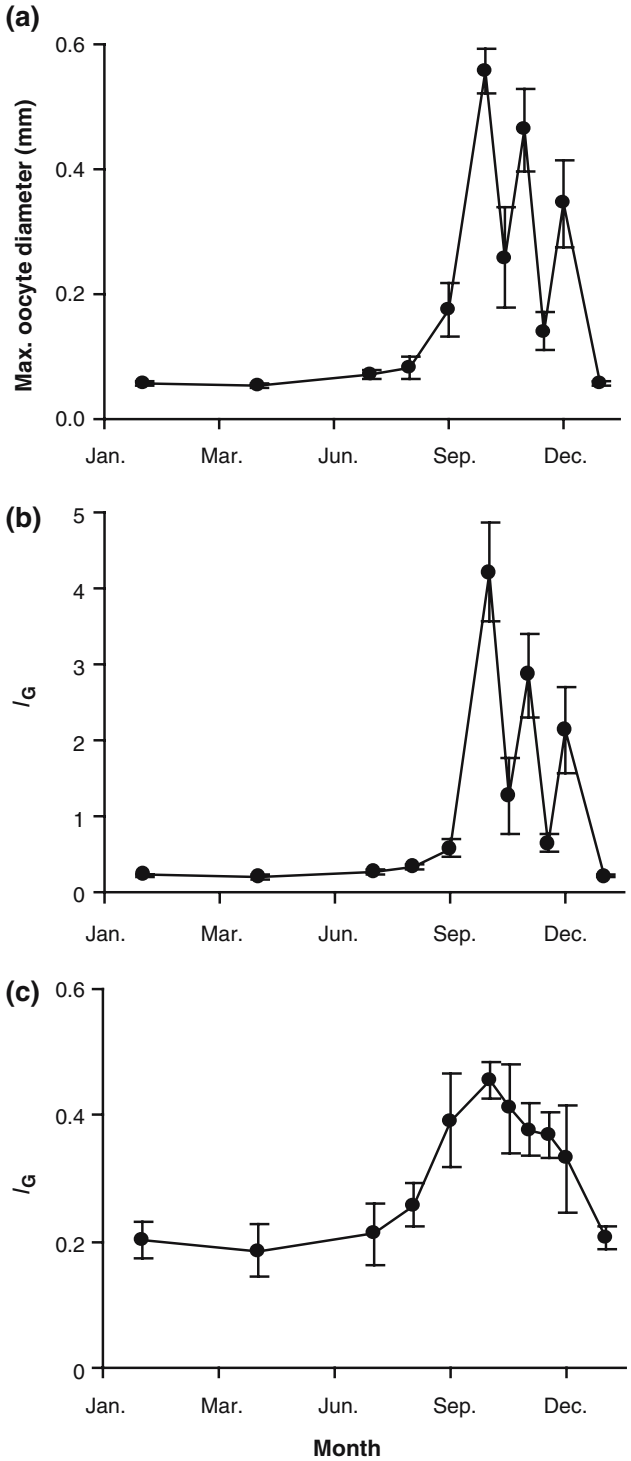

Fig. 3 Seasonal changes in maximum oocyte diameter (**a**), female gonado-somatic index (**b**) and male gonado-somatic index (**c**) of *Plectropomus leopardus* that were captured by angling on the Great Barrier Reef. See Table 1 for sample sizes

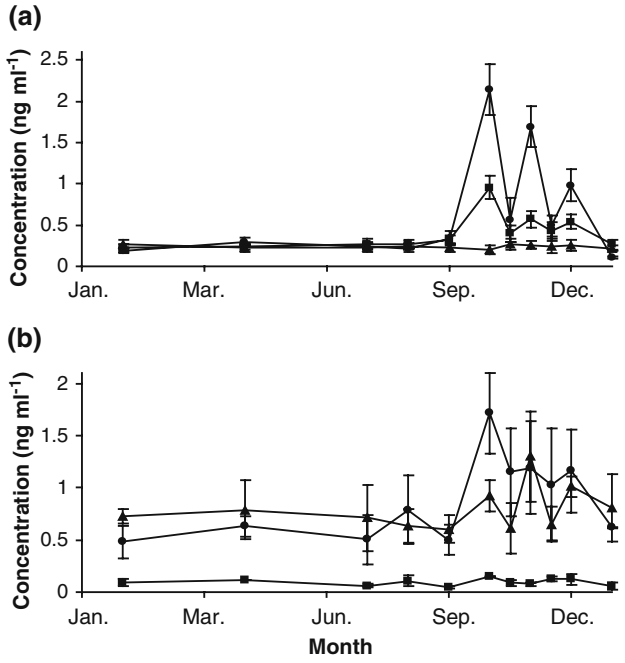

Fig. 4 Seasonal changes in plasma concentrations of estradiol-17β (*filled square*), testosterone (*filled circle*) and 11-ketotestosterone (*filled triangle*) in female (**a**) and male (**b**) *Plectropomus leopardus* that were captured by angling on the Great Barrier Reef. See Table 1 for sample sizes

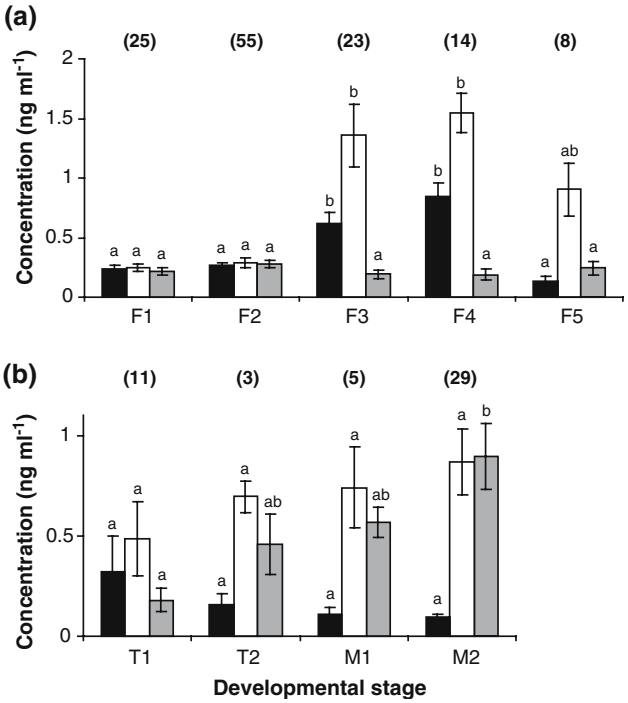

Fig. 5 Mean plasma concentrations of estradiol-17β (*black bars*), testosterone (*white bars*) and 11-ketotestosterone (*gray bars*) in female (**a**), transitional and male (**b**) *Plectropomus leopardus* that were captured by angling on the Great Barrier Reef. Numbers (in parentheses) denote sample sizes and alphabetic letters denote statistically homogenous subgroups (within each steroid hormone). See Table 2 for definitions of developmental stages

in the gonad (i.e., from T1 through to M2), while levels of E_2 declined.

Discussion

Reproductive development in *P. leopardus* was characterized by a long resting phase (late summer to winter; January–August) followed by rapid gonadal recrudescence during early spring (September–October) when water temperature commenced acclivity. However, this pattern of development was more pronounced in female fish than in male fish, presumably because of differences in the relative size of egg versus sperm. For example, maximal mean I_G for female and male fish was 4.2 and 0.46%, respectively. These values are similar to those reported for other tropical serranids [e.g., 8% for female *Epinephelus striatus*, Carter et al. (1994); 8% for female *E. merra*, Lee et al. (2002); 0.4% for male *E. morio*, Johnson et al. (1998); 0.2% for male *E. merra*, Bhandari et al. (2003)] and are typical for fishes with multiple group-synchronous patterns of gamete development (see below). In contrast, typical I_G values for synchronous species such as salmonids are commonly in the range of 20–30% (Helfman et al. 1997).

Reproductive development in female fish was cyclical during spring and early summer, strongly indicating that there were multiple ovulatory cycles and spawning episodes. Peaks in I_G, MOD and steroid hormone concentrations during this phase were synchronized with the lunar cycle. Furthermore, hydrated oocytes were found during (but not between) successive new-moon phases. Together, these results suggest that gonadal developmental in female *P. leopardus* has a lunar periodicity—a result that is consistent with previous observations of reproductive behavior in this species during new-moon phases (Samoilys and Squire 1994; Samoilys 1997). As in other fishes, lunar synchronization presumably serves to coordinate gamete maturation among conspecifics or to enhance propagule survival after spawning [reviewed by Claydon (2004) and Takemura et al. (2004)].

In contrast, male fish maintained an extended state of gonadal maturity without any apparent periodicity. In particular, spermatozoa were found in the testicular sinuses of most male fish between August and December, thus suggesting that male fish maintain the capacity to spawn throughout this period. A continuous state

of 'readiness' is typical for male vertebrates (Shuster and Wade 2003) and probably relates to the small energetic cost of producing sperm versus egg.

The presence of a wide range of oocytes at different stages of development in the ovaries of individual *P. leopardus* confirm that the pattern of gonadal development in female coral trout is multiple group-synchronous (*sensu* Pankhurst 1998) and that this species has the capacity for multiple ovulations within a single reproductive season. This notion is compatible with earlier findings that individual *P. leopardus* engage in multiple excursions to aggregation sites during spring and early summer (Zeller 1998). It is therefore apparent that individual *P. leopardus* can spawn on multiple occasions, both within a new-moon period (Samoilys and Squire 1994) and during separate new-moon periods. This type of spawning strategy may facilitate access to multiple partners, as well as maximize the chances that propagules encounter favorable conditions in an otherwise heterogeneous pelagic environment (see Doherty et al. 1985).

Despite the serial nature of gamete maturation and spawning in female *P. leopardus*, successive episodes of reproductive development may not be equivalent. Consecutive 'peaks' in the levels of I_G and MOD declined from October to December. This pattern of development suggests that the first episode of spawning is the most important in terms of reproductive investment, perhaps because endogenous energy reserves diminish with each successive spawning event. Support for this hypothesis comes from the observations that (1) plasma vitellogenin levels and I_G in rabbitfish (*Siganus guttatus*) declined during successive (lunar-synchronized) spawning cycles (Rahman et al. 2000), and (2) 'early' eggs from captive Hong Kong grouper (*Epinephelus akaara*) were larger and survived longer than eggs spawned later in the season (Kayano et al. 1998).

An alternate (but not mutually exclusive) explanation for declining peaks in gonadal activity is that small, young and (or) lean individuals of *P. leopardus* spawn only once (i.e., in October), thereby reducing the proportion of fish with spawning capacity during subsequent lunar cycles. This hypothesis is supported by the observations that (1) pre-vitellogenic female fish comprised an increasing proportion (i.e., 0, 10, and 42%) of the female fish that were collected during the new-moon phases of October, November, and December (respectively), and (2) mean I_G of vitellogenic female fish (and hydrated female fish) were unchanged during successive new-moon phases. Yet another explanation is that the time of capture changed relative to the temporal organization of gametogenesis. However, we consider this situation to be unlikely because the sampling events in both October and December (i.e., 'peaks' one and three) occurred at the same relative point in the lunar cycle (i.e., 5 days before the full moon). Furthermore, on each sampling occasion, fish were captured throughout the day without bias toward any particular time.

Before ovarian recrudescence, concentrations of E_2 and T were low and stable. During spring and early summer however, both steroids mirrored the lunar-like cycle displayed by I_G and MOD. This pattern of steroidogenesis is consistent with the putative roles of these steroids in promoting vitellogenesis (E_2) and in regulating oogenesis (E_2 and T) in other teleosts (Pankhurst and Carragher 1991; Kime 1993; Pankhurst 2006). The fact that E_2 concentrations remained elevated until oocytes became hydrated is not unusual in species where oocyte development is multiple group-synchronous (Pankhurst 2006). This is because recruitment of new oocytes (and vitellogenesis) is continuous throughout spring and early summer.

Absolute steroid concentrations in *P. leopardus* were similar to those reported for other tropical serranids (e.g., Debas et al. 1989; Johnson et al. 1998; Bhandari et al. 2003). This includes very low concentrations (or a complete absence) of E_2 and 11KT in male and female fish, respectively. This sex-specific pattern of steroid distribution therefore appears consistent among serranids [although this consistency does not extend to all teleosts: Kime (1993); Lokman et al. (2002)].

Measurable quantities of E_2, T and 11KT were found to circulate simultaneously in the plasma of transitional fish. However, absolute plasma concentrations were generally low (when compared to male or female stages), presumably to confer germ cells with the flexibility to alter their direction of development (Frisch 2004). Another emergent pattern is the concomitant increase and decrease of 11KT and E_2 (respectively) during sexual progression. Interestingly, these two steroid hormones are also the most likely candidates for the initiation and regulation of sex-change in other sequentially hermaphroditic fishes (Frisch 2004). However, due to the coarse nature of the sampling design, the role(s) of E_2 and 11KT in effectuating sex-change in *P. leopardus* remain unclear.

The overall ratio of female, transitional and male *P. leopardus* in our sample was 9:1:2.4. This is strikingly similar to the ratios reported elsewhere for this species (Ferreira 1995; Adams et al. 2000; Samoilys and Roelofs 2000), thus suggesting that our samples were indeed representative. However, skewed sex-ratios manifest as small sample sizes for transitional and male

fish, thus hindering statistical analyses on data that are specific to these stages. For example, it could not be determined when sex-change was most likely to occur, since too few transitional fish were captured. Evidence from other species however, suggests that sex-change typically occurs during the post-spawning period, when seasonal steroid levels have subsided (reviewed in Frisch 2004).

In summary, patterns of steroidogenesis and gametogenesis in *P. leopardus* were broadly similar to those reported for other tropical serranids, especially with respect to: (1) lunar periodicity, (2) the degree of reproductive investment (I_G), (3) the multiple group-synchronous mode of oocyte development, (4) the magnitude of seasonal steroid fluctuations, and (5) the evolution of steroid concentrations during sexual transition. This consistency suggests that both fisheries management regimes and broodstock propagation protocols, which have been developed for other serranids, may also be appropriate for use with *P. leopardus*. This is important considering that our knowledge of reproduction in other serranids (e.g., Nassau grouper, *E. striatus*) is generally more advanced that it is for *P. leopardus*.

Acknowledgments A. Weibken and S. Reilly assisted with histological analyses, S. Ludke assisted with steroid analyses, and M. Sheaves provided statistical advice. R. Baker, A. Kaus, N. Konow, B. McGough, J-P. Hobbs, J. Moore, L. Nankervis, P. Wruck, and S. Walker assisted in catching fish. General support was provided by K. Munkres. This work was supported by a James Cook University Doctoral Research Grant awarded to AJF and an Australian Research Council Linkage Grant awarded to NWP.

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